

Na⁺/Ca²⁺ EXCHANGE IN CARDIAC MYOCYTES

Effect of Ouabain on Voltage Dependence

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ABSTRACT Sarcolemmal sodium/calcium exchange activity was examined in individual chick embryonic myocardial cell aggregates that were loaded with quin 2. The baseline $[Ca^{2+}]_i$ was 68 ± 4 nM ($n = 29$). Abrupt superfusion with sodium-free lithium solution produced a fourfold increase in steady-state $[Ca^{2+}]_i$ to 290 ± 19 nM, which was reversible upon sodium restitution. Other methods of increasing $[Ca^{2+}]_i$ such as KCl-depolarization or caffeine produced a dose-dependent increase in quin 2 fluorescence, accompanied by sustained contracture. The $[Ca^{2+}]_i$ increase in zero sodium was linear, and its half-time ($t_{1/2}$) of 15.1 ± 0.1 s was similar to that of the sodium-free contracture ($t_{1/2} = 14.4 \pm 0.5$ s) under the same conditions. The sodium-dependent $[Ca^{2+}]_i$ increase was not significantly greater when potassium served as the sodium substitute instead of lithium. This suggests that sodium/calcium exchange has little voltage dependence in this situation. However, in aggregates pretreated with ouabain (2.5 μ M), the $[Ca^{2+}]_i$ increase was almost threefold greater with potassium than with lithium ($P < 0.007$). Ouabain therefore potentiated the effect of membrane potential on calcium influx. We propose that elevation of $[Na^+]_i$ is a prerequisite for voltage dependence of the sodium/calcium exchange under the conditions studied. Sodium loading will then drastically increase calcium influx during the action potential while inducing an outward membrane current that could accelerate repolarization.

INTRODUCTION

More than fifteen years have elapsed since a sodium/calcium exchange mechanism was first demonstrated in squid giant axon (1) and in heart (2). Despite increasing interest, the properties and functions of the sodium/calcium exchange remain speculative (3–5). While there is substantial evidence for voltage dependence and electrogenicity, the exact stoichiometry remains uncertain (3). The sodium/calcium exchange is thought to regulate myocardial contractile states by providing external Ca^{2+} during depolarization and extruding intracellular Ca^{2+} during rest, but the ability of the exchange to reverse during membrane depolarization has not been established. Evidence for both inwardly and outwardly directed sodium/calcium exchange currents have been obtained, but the exact conditions under which these currents occur have not been defined (6–9). A major impediment to the investigation of these phenomena has been the technical difficulty of measuring intracellular free calcium, $[Ca^{2+}]_i$. Older calcium measurement techniques are especially challenging in cardiac cells (10) and have produced conflicting results in certain experimental conditions (11).

Recently, nondisruptive measurements of $[Ca^{2+}]_i$ have become possible using a new class of calcium-specific fluorescent indicators, the prototype of which is quin 2 (12–14). Quin 2 is a fluorescent tetracarboxylic acid that is

highly selective for calcium, binding Ca^{2+} with 1:1 stoichiometry. Introduction of quin 2 into cells is achieved by incubation with quin 2 AM, the acetoxymethyl ester of quin 2. The quin 2 fluorescence signal (excitation, 339 nm; emission, 492 nm) is enhanced fivefold on binding with Ca^{2+} . Mean cytoplasmic calcium activity can therefore be determined through calibration of cellular fluorescence. This technique has been used to measure $[Ca^{2+}]_i$ in cell suspensions from several different sources (13–20).

We report here a microfluorometric technique for measuring $[Ca^{2+}]_i$ in individual chick embryonic myocardial cell aggregates loaded with quin 2. This arrangement permits changes in $[Ca^{2+}]_i$ to be monitored during rapid alteration of the extracellular ionic environment. Using this system, we have studied the effects of abrupt sodium removal on $[Ca^{2+}]_i$ under a variety of conditions that would be expected to modulate the resulting calcium influx. Our results indicate that sodium-dependent calcium influx is strongly enhanced by depolarization under conditions of internal sodium loading, but is not particularly voltage-dependent in the absence of sodium loading. We conclude that intracellular sodium modifies the stoichiometry or kinetics of the sodium/calcium exchange in a manner that confers voltage dependence and electrogenicity. This mechanism would increase the dependence of contractile force on $[Na^+]_i$ (21), and would modulate outward sodium/calcium exchange current in a manner that is consis-

tent with the known effects of sodium loading on action potential duration (21). Some of our results have been described in a preliminary communication (22).

MATERIALS AND METHODS

Ventricles were obtained from 9–12-d chick embryos by sterile dissection. Cells were dissociated by multiple-cycle trypsinization (24). Ventricular fragments from 15–20 hearts were immersed in 0.05% trypsin (Nutritional Biochemicals Corp., Cleveland, OH) at 37°C and agitated gently by a mechanical stirring bar for 7 min/cycle for three to four cycles. Dispersed cells were pooled, suspended in culture medium, and injected into Sykes Moore chambers (Bellco Glass, Inc., Vineland, NJ) at a density of 1×10^6 /ml. The bottom of each chamber was coated with a thin layer of Sylgard resin (Dow Corning Corp., Midland, MI), which prevented attachment of cells. Isopotential aggregates formed within 2–3 d and were maintained in culture until the time of the experiment (up to 7 d) when they were transferred to a resin-free glass surface. The cell culture medium contained 20% nutrient medium M-199 (Gibco, Grand Island, NY), 6% heat-inactivated fetal calf serum (HyClone Laboratories, Sterile Systems, Inc., Logan, UT), 1% carbenicillin-gentamicin solution, and 73% potassium-free salt solution, the composition of which was 116 mM NaCl, 26.2 mM NaHCO₃, 0.08 mM MgSO₄, 0.9 mM NaH₂PO₄, 1.8 mM CaCl₂, and 5.5 mM dextrose, adjusted to pH 7.4.

Experiments were performed in physiological saline containing 137 mM NaCl, 0.4 mM NaH₂PO₄, 2.7 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, 5.5 mM dextrose, and 6.0 mM HEPES at pH 7.4 and $37 \pm 1^\circ\text{C}$. Sodium-free solutions had the same composition as the HEPES-buffered physiological saline except that sodium salts were replaced by lithium or potassium salts. Small amounts of Tris-base were used to adjust the pH of the sodium-free lithium solution. High potassium solutions that contained sodium were isotonic and were prepared by partial sodium replacement. Superfusion of cell aggregates was performed using a 270- μm (internal diameter) polyethylene catheter, positioned $\sim 100 \mu\text{m}$ from the aggregate and connected to a syringe pump. Abrupt superfusion at a flow rate of 350 $\mu\text{l}/\text{min}$ produced complete exchange of solute over a region several times larger than an aggregate's diameter within 1–2 s.

Cell aggregates were loaded with quin 2 by incubation with 30–50 μM of quin 2 AM in tissue culture medium for 60 min (13, 14). Extracellular quin 2 AM was then removed by washing with HEPES-buffered physiological saline. Cells thus loaded were expected to contain 0.5–1.0 mM of trapped intracellular quin 2, which did not noticeably affect contraction. Quin 2 recordings were obtained in a nonfluorescent chamber on the stage of an inverted microscope. Spheroidal aggregates 80–200 μm in diameter, which contain 300–2,600 cells, were selected for the study. A 100 W ultraviolet source (E. Leitz, Inc., Rockleigh, NJ), with a $340 \pm 5 \text{ nm}$ interference filter (Corion Corp., Holliston, MA), was directed at an oblique angle onto the cells. The fluorescence signal was measured by a photomultiplier tube (model IP21; Hamamatsu Corp., Middlesex, NJ) placed in the focal plane of a camera port. Light reaching the photomultiplier passed through a $500 \pm 20 \text{ nm}$ filter, the transmission frequency of which encompassed the quin 2 fluorescence peak. The photomultiplier output was electronically filtered using a lowpass active filter (model 901F; Frequency Devices, Inc., Haverhill, MA) and was recorded by a strip-chart recorder.

Minimum (F_{\min}) and maximum (F_{\max}) fluorescence of the quin 2-loaded aggregates were determined at the end of the experiment after all measurements of cellular fluorescence were completed. Each aggregate was first exposed to a solution containing 20 mM CaCl₂ and 150 nM ionomycin. The presence of ionomycin allowed extracellular calcium to enter the cells and saturate the quin 2 to give F_{\max} . The aggregate was then exposed to a solution containing 150 nM ionomycin and 10 mM MnCl₂. Entry of Mn²⁺ into cells was also mediated by ionomycin and quenched quin 2 fluorescence due to its higher affinity for quin 2 binding (23). F_{\min} , the quin 2 fluorescence in the absence of Ca²⁺, was then given by (23)

$$F_{\min} = F_{\text{Mn}^{2+}} + 0.16(F_{\max} - F_{\text{Mn}^{2+}}). \quad (1)$$

The concentration of intracellular free calcium $[\text{Ca}^{2+}]_i$ was then calculated according to Tsien (14)

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\min})/(F_{\max} - F), \quad (2)$$

where F was the steady-state cellular fluorescence measured, $F_{\text{Mn}^{2+}}$ (in Eq. 1) was the fluorescence measured in the presence of MnCl₂, and K_d was 115 nM.

In some experiments, contractile responses of quin 2-loaded myocardial cell aggregates were studied by recording cell edge movement with a photodiode placed in the image plane of an eyepiece lens. The photodiode output has been shown to be linearly correlated with edge displacement, which is increased by positive inotropic interventions such as ouabain treatment or depolarizing current pulses (24).

Unless otherwise stated, at least five determinations were obtained for each experimental condition. Two types of controls were used. First, each experiment was repeated in two to four different aggregates not containing quin 2. Second, quin 2-loaded cell aggregates were superfused with physiological saline, which did not affect their fluorescence. Group data were expressed as mean \pm SE. Statistical significance was determined by Student's t test (two-tailed distribution).

RESULTS

Sensitivity of Quin 2 to Increased Cytoplasmic Calcium

The purpose of our initial experiment was to determine the steady-state intracellular calcium activity, $[\text{Ca}^{2+}]_i$, in quin 2-loaded myocardial cell aggregates. The resulting value was $68 \pm 4 \text{ nM}$ in 29 aggregates. This figure agrees with the value reported in suspensions of chick embryonic myocardial cells (20), although it is somewhat lower than values obtained in cells from adult mammalian hearts (18, 19).

Superfusion with a sodium-free lithium solution produced a concomitant increase in cellular fluorescence. Cessation of superfusion, which restored external sodium, was accompanied by return of cellular fluorescence to the baseline value. Resulting fluorescence profiles are shown in Figs. 1 and 2. The steady-state $[\text{Ca}^{2+}]_i$ typically increased about fourfold to $290 \pm 19 \text{ nM}$ in sodium-free lithium solution ($n = 8$, $P < 10^{-7}$). No detectable increase in cellular fluorescence was noted when the experiment was

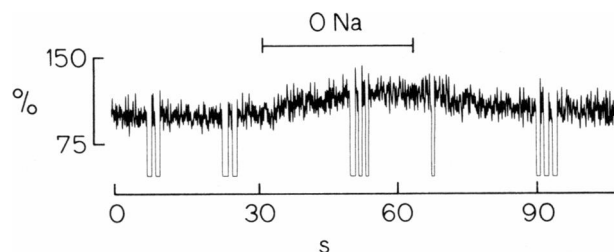


FIGURE 1 Fluorescence profile obtained in a quin 2 loaded myocardial cell aggregate superfused with sodium-free LiCl solution. Results are expressed as percent resting fluorescence. Zero fluorescence is obtained when a shutter on the UV illuminator is closed (shown as interruptions in the fluorescence signal). Superfusion with sodium-free LiCl solution (horizontal bar) produces a progressive increase in cellular fluorescence. Cessation of superfusion, which restores extracellular sodium, causes return of fluorescence to the baseline.

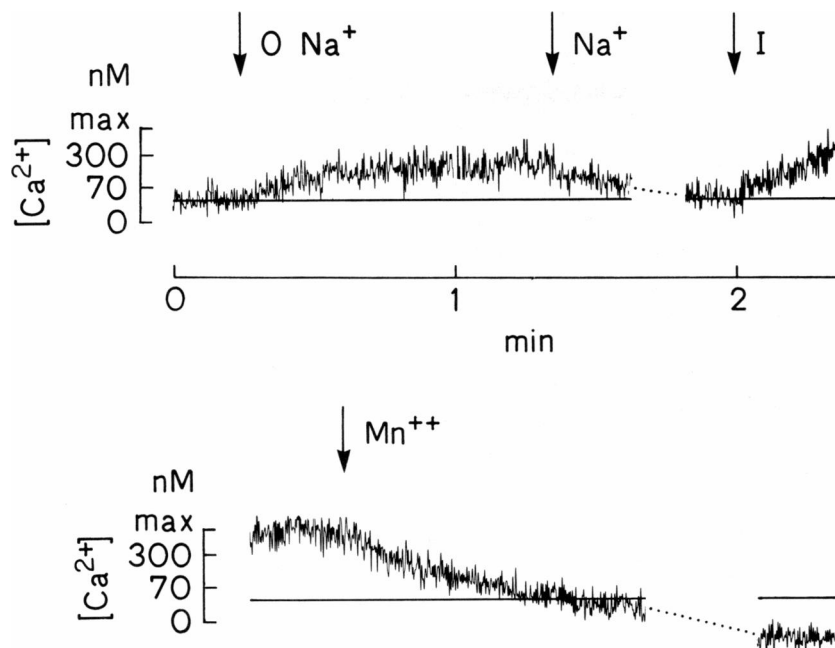


FIGURE 2. Measurements of $[Ca^{2+}]_i$ in a quin 2 loaded myocardial cell aggregate superfused with sodium-free LiCl solution. Baseline fluorescence is obtained with the aggregate in HEPES-buffered physiological saline. Sodium removal (first arrow, top) produces a progressive increase in cellular fluorescence that takes 25–30 s to reach steady-state. Restitution of sodium (second arrow) is followed by return of fluorescence to the baseline. $[Ca^{2+}]_i$ is calibrated by first exposing the aggregate to 150 nM ionomycin and 20 mM $CaCl_2$ (third arrow), which saturates the quin 2 signal giving F_{max} (bottom, left). The aggregate is then exposed to 150 nM ionomycin and 10 nM $MnCl_2$ (last arrow), which quenches quin 2. $[Ca^{2+}]_i$ is calculated according to Eqs. 1 and 2 in the text. Resulting values increase from 64 to 265 nM during sodium-free superfusion. The signal-to-noise ratio is superior to Fig. 1 because of improvements in the recording system.

repeated in cell aggregates not loaded with quin 2, or when quin 2-loaded cells were superfused with physiological saline.

Other methods of increasing $[Ca^{2+}]_i$ also produced corresponding increases in the fluorescence signal. On exposure to 30 mM, 60 mM, and 90 mM KCl, the steady-state $[Ca^{2+}]_i$ increased to 170 ± 3 , 250 ± 18 , and 586 ± 107 nM, respectively (Table I). Superfusion of quin 2 loaded cells with a nominally calcium-free KCl solution produced no detectable increase in cellular fluorescence, confirming the calcium-dependence of the quin 2 signal.

Depending upon its distribution, quin 2 might also detect release of calcium from intracellular compartments. Accordingly, quin 2-loaded cells were superfused with 10 mM caffeine, which is known to release calcium from the sarcoplasmic reticulum (25). Caffeine exposure produced a more than twofold increase in steady-state $[Ca^{2+}]_i$ to 175 ± 11 nM (Table I). This cytoplasmic calcium increase was not blocked by 1 mM $CoCl_2$. Hence, the ability of quin 2 to detect increased calcium was independent of the route of calcium entry.

Correlation of Quin 2 Fluorescence with Contractile Response

To further confirm the relationship between quin 2 fluorescence and $[Ca^{2+}]_i$, we compared the fluorescence increase with accompanying contractures. Contractile displace-

TABLE I
EFFECT OF ZERO-NA, KCl, AND CAFFEINE ON $[Ca^{2+}]_i$ IN QUIN 2 LOADED MYOCARDIAL CELL AGGREGATES

Condition	Steady-state $[Ca^{2+}]_i$ * nM
Baseline	68 ± 4 ($n = 29$)
Sodium-free LiCl solution	290 ± 19 ($n = 8, P < 10^{-7}$)
30 mM KCl	170 ± 3 ($n = 5, P < 10^{-7}$)
60 mM KCl	250 ± 18 ($n = 6, P < 10^{-5}$)
90 mM KCl	586 ± 107 ($n = 5, P = 0.002$)
10 mM Caffeine	175 ± 11 ($n = 6, P < 10^{-5}$)

Values are mean \pm SE, while n is the number of measurements. P values are obtained using baseline measurements for each subgroup. Diltiazem is absent here, which explains why the value for 90 mM KCl exceeds that in Fig. 6 A (control point).

*Since submission of our paper, Lattanzio and Pressman (47) have proposed that the K_d for quin 2 in cardiac cells is higher than 115 nM, based on recent measurements of intracellular Mg^{2+} . If we recalculate $[Ca^{2+}]_i$ using their K_d of 240 nM, the values increase by a factor of 2.1. For example, the mean baseline $[Ca^{2+}]_i$ becomes 142 ± 8 nM. This value is similar to those obtained in Purkinje fibers with ion selective electrodes (e.g., reference 48), and to values we obtain in cell aggregates using the second generation indicator, indo 1.

ment of the cell edge was measured by the optical transmittance technique used previously (24). The mechanical response of a quin 2 loaded aggregate to sodium removal is illustrated in Fig. 3 *A*. Abrupt exposure to sodium-free lithium solution (arrow), caused a large sustained contracture, which typically developed in 20–30 s. The time course of this contracture was similar to that observed in aggregates without quin 2 (26). Restoration of external sodium (second arrow) was accompanied by relaxation. The half-time of contracture development in lithium ($t_{1/2} = 14.4 \pm 0.5$ s, $n = 7$) was similar to that of the $[Ca^{2+}]_i$ increase ($t_{1/2} = 15.1 \pm 0.1$ s).

Similar agreement between the contracture and fluorescence signals was obtained during exposure to KCl. In contrast to the contractures in LiCl solution, those induced by 60 mM KCl reached steady state within 3–5 s (Fig. 3 *B*, arrow). Recordings of quin 2 fluorescence in high potassium also reached steady state rapidly (within 5–10 s), presumably because of rapid calcium entry through voltage-dependent channels.

Linearity of the Sodium-Dependent Calcium Increase

The time course of the $[Ca^{2+}]_i$ increase could be derived from the fluorescence signal using Eq. 2. The $[Ca^{2+}]_i$ increase in a single aggregate exposed to sodium-free LiCl solution (shown in Fig. 4 *A*) was linear ($R = 0.99$), with a slope of 7.4 nM/s. Similar plots obtained in five aggregates have been displayed in normalized form in Fig. 4 *B*. These plots were also linear ($R = 0.98$) and gave a corresponding mean rate of $6.8 \text{ nM} \pm 1.0 \text{ nM/s}$ ($n = 5$). The linearity of the $[Ca^{2+}]_i$ increase may have physiological significance. Sodium-dependent uptake of radioactive $^{45}Ca^{2+}$ by cardiac

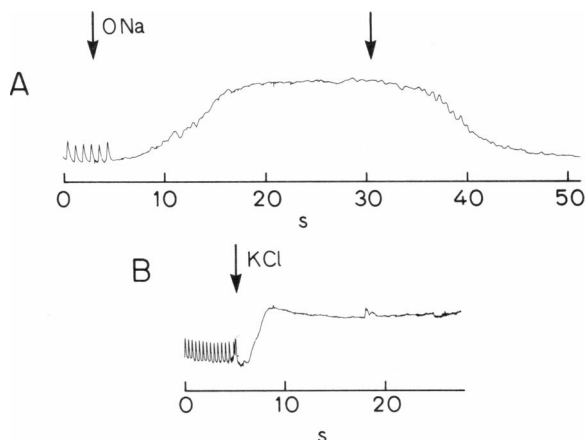


FIGURE 3 Optical recordings of contractile activity in quin 2 loaded myocardial cell aggregates. In *A*, sodium-free LiCl solution is applied between the arrows. Sodium removal causes immediate inhibition of spontaneous activity, followed by a sustained contracture. The half-time of contracture development (10.5 s) is similar, though somewhat shorter than typically observed. In *B*, a different aggregate is exposed to 60 mM KCl. Development of the contracture is faster in KCl than in any of five aggregates exposed to sodium-free LiCl solution.

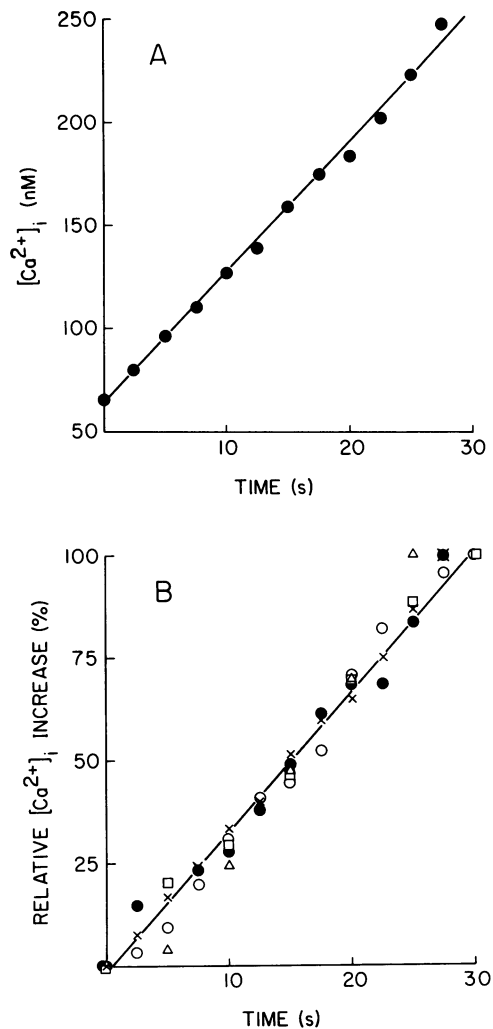


FIGURE 4 Time course of the $[Ca^{2+}]_i$ increase during exposure of quin 2 loaded myocardial cell aggregates to sodium-free LiCl solution. *A* shows results from a single aggregate. The $[Ca^{2+}]_i$ increase is linear ($R = 0.99$), with a rate of 7.4 nM/s. *B* shows normalized data from five similar experiments. The baseline value is 0, while the maximum increase is 100%. The resulting plot is also linear ($R = 0.98$) and its slope corresponds to a $[Ca^{2+}]_i$ increase of $6.8 \pm 0.4 \text{ nM/s}$. The half-time ($t_{1/2}$) of the $[Ca^{2+}]_i$ increase is 15.1 ± 0.1 s.

sarcolemmal vesicles is also reportedly linear during the first 20–30 s (27, 28) and in this situation, a constant velocity of sodium/calcium exchange can be inferred. The increase in $[Ca^{2+}]_i$ levels off after 30 s, reflecting either a reduction in calcium influx, or increased extrusion.

Modulation of Sodium/Calcium Exchange by Sodium-Loading and Depolarization

The rate of sodium-dependent calcium influx might be expected to vary with internal sodium concentration. In addition, depending upon the stoichiometry, the rate of calcium influx could vary with membrane potential as well. To assess the effect of sodium loading, the steady-state quin 2 fluorescence was measured in cell aggregates that were pretreated with $2.5 \mu\text{M}$ ouabain for 2 min before

sodium removal. Ouabain concentrations in this range have been shown to produce a severalfold increase in $[Na^+]_i$ (29). The effect of membrane depolarization was studied by exposing the cells to a solution in which potassium rather than lithium was used as the sodium substitute. Exposure of quin 2-loaded cells to sodium free KCl solution produced a steady resting potential of -4.6 ± 0.9 at 15 s, compared with a value of -42.8 ± 1.0 mV in LiCl solution ($P < 10^{-7}$). Results were similar to ouabain treated aggregates (-4.5 ± 0.8 mV in KCl vs. -52.8 ± 1.9 mV in LiCl; $P < 10^{-6}$), though the LiCl values were somewhat more negative. For these experiments, the cell aggregates were pretreated with diltiazem (0.1 mM), which reduces calcium entry through voltage-gated channels.

The effects of sodium loading and membrane potential on quin 2 fluorescence are summarized in Fig. 6. The steady-state $[Ca^{2+}]_i$ on exposure to sodium-free LiCl solution increased from a baseline of 63 ± 8 nM to 277 ± 18 nM ($P < 0.001$; $n = 10$). The presence of 0.1 mM diltiazem had no discernible effect on the lithium response. Pretreatment with ouabain ($2.5 \mu M$ for 2 min) before LiCl caused a slight and statistically insignificant increase in the steady-state $[Ca^{2+}]_i$ (to 311 ± 19 nM, $n = 7$). Similarly, the use of KCl instead of LiCl as the sodium substitute caused a minor increment in steady-state $[Ca^{2+}]_i$ (to 304 ± 41 nM, $n = 8$). Therefore, neither depolarization nor sodium loading alone had a marked effect on sodium-dependent calcium influx. However, when ouabain-pretreated cell aggregates were exposed to sodium-free KCl solution, a dramatic increase in the fluorescence signal was observed (Fig. 5). The steady-state $[Ca^{2+}]_i$ achieved in this situation was 757 ± 129 nM ($n = 8$). This $[Ca^{2+}]_i$ increase was almost threefold larger than the increase observed in ouabain-treated aggregates exposed to sodium-free lithium solution ($P < 0.007$); or in nonouabain-treated cells exposed to sodium-free potassium solution ($P < 0.005$).

The overall conclusion from Fig. 6 is that sodium loading potentiates the effect of membrane potential on

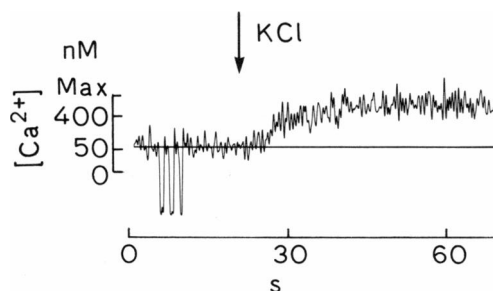


FIGURE 5 Effect of sodium-free KCl solution on $[Ca^{2+}]_i$ in a sodium loaded cell aggregate. The aggregate is pretreated with $2.5 \mu M$ ouabain and 0.1 mM diltiazem 2 min before superfusion. Exposure to sodium-free KCl solution (arrow) induces a more than sevenfold increase in $[Ca^{2+}]_i$. Note that the initial rate of $[Ca^{2+}]_i$ increase is much faster than in sodium-free LiCl solution (e.g., Fig. 2).

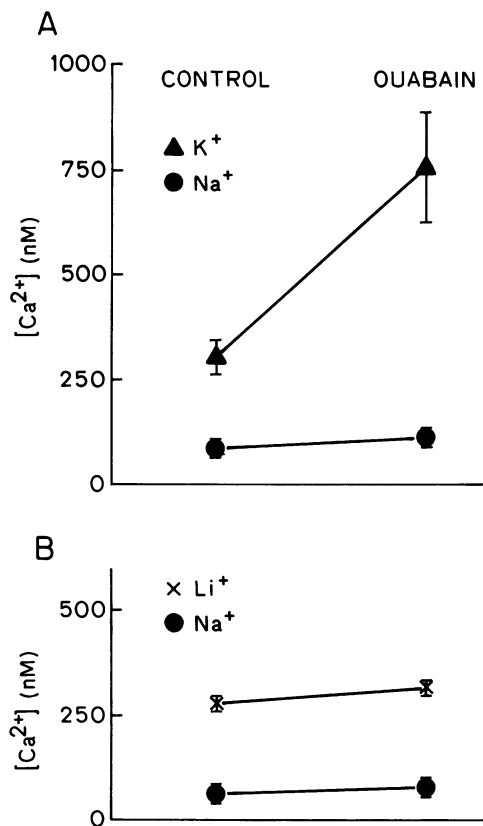


FIGURE 6. Synergistic effect of intracellular sodium loading and depolarization on the sodium-dependent $[Ca^{2+}]_i$ increase. (A) Results from two groups of aggregates exposed to sodium-free KCl solution after pretreatment with 0.1 mM diltiazem. A control group (left hand points, $n = 8$) has been pretreated with diltiazem alone, while a second group (right hand points, $n = 8$) is pretreated with diltiazem plus $2.5 \mu M$ ouabain. The steady-state $[Ca^{2+}]_i$ on superfusion with KCl is much higher with ouabain pretreatment (757 ± 129 nM, $n = 8$) than without (304 ± 41 nM, $n = 8$, $P = 0.005$). (B) a similar experiment in which LiCl serves as the sodium substitute. The steady-state $[Ca^{2+}]_i$ on superfusion with sodium-free LiCl is not significantly different between the ouabain group (311 ± 19 nM, $n = 7$) and the control group (277 ± 18 nM, $n = 10$, $P = 0.22$). The $[Ca^{2+}]_i$ increase in zero sodium is highly significant in all four groups of experiments ($P = 0.0002$ – 10^{-9}). The four baseline values of $[Ca^{2+}]_i$ (filled circles) do not differ significantly, even in the presence of ouabain. However, higher concentrations of ouabain can increase $[Ca^{2+}]_i$ in the absence of other interventions (cf. references 18–20).

sodium-dependent calcium influx. This result suggests a novel mechanism by which ouabain and other agents may increase calcium influx during depolarization.

DISCUSSION

The concept of a sodium/calcium exchange was first introduced by Reuter and Seitz in 1968 through observations of calcium flux measurements in guinea pig atria (2). Since then, a sodium-dependent calcium countertransport system has been shown to exist in a variety of tissues and preparations, including squid giant axons, barnacle muscle fibers, and cardiac muscle (1, 4, 30). In cardiac myocytes, the function of the sodium/calcium exchange remains

speculative (5, 30), but it is believed to be important in two situations. The first of these is the maintenance of a low $[Ca^{2+}]_i$. Intracellular calcium is extruded during rest through exchange with extracellular Na^+ , the energy of this exchange being derived from the Na^+/K^+ ATPase. Second, if the exchange has appropriate stoichiometry, it will reverse during depolarization, providing net calcium influx during the plateau phase of action potential. The sodium/calcium exchange could therefore be a significant source of calcium during phasic contractions.

The stoichiometry and other properties of the sodium/calcium exchange have been studied extensively through flux measurements employing sarcolemmal vesicles and intact cells (27, 28, 31–38). While an exact stoichiometry has not been established, there is overwhelming evidence for voltage dependence and electrogenicity during both calcium influx and efflux (3–9, 28, 33–41). It is significant, however, that the evidence for electrogenic calcium influx derives from experiments involving supra-physiologic sodium loads, often achieved through sodium pump inhibition (8, 28, 34, 40, 41).

Our report examines the factors governing sodium-dependent calcium influx, as deduced from measurements of mean cellular calcium activity obtained with quin 2. Abrupt sodium removal causes a highly reproducible increase in $[Ca^{2+}]_i$, which has a time course similar to that of the sodium-free contracture. The time course of the calcium increase in sodium-free lithium solution is also similar to that of the calcium-activated nonspecific current, which has been described in voltage-clamp experiments (26). The contracture, the calcium increase, and the calcium-activated current all decay with a similar time course when sodium is restored (Figs. 1–3; reference 26).

It is important to recognize that the increase in mean calcium activity measured by quin 2 cannot automatically be equated with calcium influx. The relation between calcium uptake and quin 2 fluorescence may be complicated by a number of factors including: (a) Sequestration of intracellular calcium within membrane-bound compartments, (b) unequal distribution of quin 2 among the intracellular compartments, (c) buffering of calcium by intracellular proteins, and (d) buffering of calcium by quin 2. However, in spite of these factors, a reproducible and monotonic relationship between calcium influx and measured calcium activity is still expected.

Several of our observations confirm that the relationship between calcium uptake and $[Ca^{2+}]_i$ is monotonic, if not actually linear. First, maneuvers or combinations of maneuvers that promote calcium entry produce a continuous variation in steady-state $[Ca^{2+}]_i$ over a 10-fold range (Table I). The similar time course of the quin 2 fluorescence increase, the mechanical contracture, and the calcium-activated conductance increase (26) also argue against nonlinearity of the quin 2 response. Finally, the fact that $[Ca^{2+}]_i$ in our experiments (Fig. 4) and $^{45}Ca^{2+}$ uptake in sarcolemmal membrane vesicles both increase linearly

during the first 20–30 s in low sodium (27, 28) corroborates the proportionality between calcium influx and $[Ca^{2+}]_i$. A constant rate of influx would be expected in this situation, since extracellular calcium binding sites will be saturated (35), while the back reaction (sodium-dependent calcium efflux) is blocked.

Having available a quantitative indicator of calcium uptake, we proceeded to study the effects of sodium loading and depolarization on sodium/calcium exchange. We confirm that, in the presence of increased internal sodium, sodium-dependent calcium influx is markedly voltage-sensitive, producing a 2.7-fold greater increase in $[Ca^{2+}]_i$ during KCl-induced depolarization than during sodium removal alone. However, with normal intracellular sodium, the sodium/calcium exchange exhibits minimal voltage sensitivity. This is a provocative finding because it suggests that the voltage dependence of the sodium/calcium exchange, and hence its electrogenicity, may be regulated by intracellular sodium activity. Our findings agree with recent studies in voltage-clamped dialyzed squid giant axons, in which the voltage sensitivity of sodium/calcium exchange was also increased by sodium loading (36, 37). However, the effects of $[Na^+]_i$ on the voltage-dependence of the cardiac sodium/calcium exchange have not previously been reported.

How internal sodium regulates the voltage dependence of the sodium/calcium exchange remains unclear. One possible explanation is that the stoichiometry of the exchange is variable. At low $[Na^+]_i$, the exchange may be almost electroneutral (stoichiometry close to 2:1) and therefore minimally sensitive to voltage. However, as $[Na^+]_i$ increases, the transport ratio could increase (stoichiometry of 3 or 4:1), so that voltage sensitivity would develop. Binding of the third sodium ion to the carrier would then be an optional event, the probability of which depends on $[Na^+]_i$. The alternative possibility is that sodium binds to an allosteric site on the carrier, which alters its voltage sensitivity without changing the stoichiometry. The allosteric site might, for example, determine whether a voltage-dependent reaction step is rate limiting. In this case, the carrier could lose its voltage sensitivity, but still be electrogenic, when $[Na^+]_i$ is low.

The observation that sodium loading is a prerequisite for voltage dependence of the exchange has far-reaching physiological implications. First, it explains why the precise stoichiometry of the exchange has been so elusive. Second, it explains the exquisite sensitivity of depolarization-induced calcium entry to $[Na^+]_i$. Third, it predicts that an increase in $[Na^+]_i$ can cause the appearance of an outward membrane current which will accelerate repolarization of the action potential.

An inverse relation between action potential duration and $[Na^+]_i$ is well established under a variety of conditions including sodium pump inhibition and variation of heart rate. The ability of digitalis to shorten the action potential is counteracted by adriamycin, a putative inhibitor of

sodium/calcium exchange (42). We have measured the effect of 1 μ M ouabain on the action potential in myocardial cell aggregates and find that the duration at 50% repolarization (APD₅₀) is reduced by 29% \pm 5% within 2 min. Thus, the acquisition of voltage dependence by the sodium/calcium exchange is associated with significant reduction of plateau duration. The role of sodium/calcium exchange during rapid stimulation is supported by direct measurements of [Na⁺]_i (21, 43). There is an impressive temporal correlation between the increase in intracellular sodium activity, the increase in phasic tension and the abbreviation or "downward" displacement of the action potential plateau (21, 44–46).

Of course, sodium/calcium exchange could also regulate contractile force and membrane potential if its kinetics and stoichiometry were independent of [Na⁺]_i. However, it is difficult for such a scheme to account for the dramatic responses that small variations in [Na⁺]_i appear to produce. For example, when short depolarizing stimulus trains are applied to sheep Purkinje fibers, a 1 mM increase in [Na⁺]_i is associated with a twofold increase in contractile force (21). Such a marked effect is consistent with our proposed model, in which sodium loading would activate a dormant pathway for calcium influx during depolarization.

Further evidence regarding the physiological role of sodium/calcium exchange can probably be obtained by recording [Ca²⁺]_i in intact cardiac cells. However, studies involving purification or reconstitution of the exchanger may be necessary to identify the molecular mechanisms that determine its voltage dependence under various conditions (31).

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